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(54) **Method for producing L-amino acid by fermentation**

(57) L-amino acid such as L-threonine and L-glutamic acid is produced by cultivating a bacterium belonging to the genus *Escherichia* which is transformed with a gene coding for pyruvate carboxylase and has L-amino acid productivity; producing and accumulating L-amino acid in the medium and collecting the L-amino acid from the medium.

EP 1 092 776 A1

Description

Technical Field

5 [0001] The present invention relates to a method for producing L-amino acid, specifically relates to a method for producing L-threonine, L-glutamic acid, L-homoserine, L-methionine, L-arginine, L-proline or L-isoleucine using a bacterium belonging to the genus *Escherichia*.

Background Art

10 [0002] L-amino acids such as L-threonine and L-glutamic acid have conventionally been produced by fermentation methods principally utilizing coryneform bacteria which belong to the genera *Brevibacterium*, *Corynebacterium*, and *Microbacterium* or mutants thereof ("Amino Acid Fermentation", Gakkai Shuppan Center, pp.195-215, 1986).

[0003] On the other hand, there has been disclosed techniques for breeding of L-amino acid-producing bacteria 15 belonging to the genus *Escherichia* utilizing genetic recombination techniques.

[0004] For example, there has been disclosed a method of producing L-lysine using *Escherichia coli* in which genes coding for dihydrodipicolinate synthetase, aspartokinase are desensitized to feedback inhibition by L-lysine and L-threonine, and diaminopimelate dehydrogenase (or tetrahydrodipicolinate succinylase and succinyldiaminopimelate deacylase) are enhanced (WO 95/16042).

20 [0005] Though the productivity of L-amino acids has considerably been improved by breeding of such microorganisms as mentioned above or production processes have been improved, it is still desired to develop more efficient processes for producing L-amino acids in order to meet the expected markedly increased future demand of the amino acids.

[0006] Pyruvate carboxylase (pyruvate:carbon dioxide ligase (ADP-forming), EC 6.4.1.1] is a biotine containing enzyme. It catalyses the carboxylation of pyruvate to form oxaloacetate. Pyruvate carboxylase has not been known in 25 *Escherichia coli*, but *Bacillus subtilis* (Diesterhaft, M.D. and Freese, E., J. Biol. Chem., 248, No.17, 6062-6070 (1973)) and *Corynebacterium glutamicum* (Peters-Wendisch, P. G. et al., *Microbiology*, 144, 915-927 (1998)) have pyruvate carboxylase and the nucleotide sequences of the genes coding for the enzymes have been reported.

[0007] Further, there has been known *Corynebacterium glutamicum* in which an activity of pyruvate carboxylase is enhanced or *Corynebacterium glutamicum* of which pyruvate carboxylase gene is inactivated (Peters-Wendisch, P. G. 30 et al., *Microbiology*, 144, 915-927 (1998)). However, these microorganisms were created as experimental materials to study enzymes necessary for growth with glucose. The relation between the activity of pyruvate carboxylase and productivity of L-amino acids has not been known. Also, there has not been known an attempt to introduce the pyruvate carboxylase gene into a bacterium belonging to the genus *Escherichia*.

Summary of the Invention

[0008] The present invention has been accomplished from the aforementioned point of view, and its object is to provide a method for producing L-amino acid, especially L-threonine, L-lysine, L-glutamic acid, L-homoserine, L-methionine, L-arginine, L-proline or L-isoleucine in high efficiency.

40 [0009] As a result of diligent investigation in order to achieve the object described above, the present inventors have found that introduction of a gene coding for pyruvate carboxylase (hereinafter referred to as "pyc gene") into a bacterium belonging to the genus *Escherichia* can improve L-amino acid productivity of the bacterium. Thus, the present invention has been completed.

[0010] That is, the present invention is a bacterium belonging to the genus *Escherichia* which is transformed with 45 a gene coding for pyruvate carboxylase and has L-amino acid productivity.

[0011] The present invention also provides the bacterium described above wherein the gene coding for pyruvate carboxylase is derived from a bacterium belonging to the genus *Bacillus*.

[0012] The present invention further provides the bacterium described above wherein the gene coding for pyruvate carboxylase is introduced into said bacterium in a low copy number.

50 [0013] The present invention still further provides the bacterium described above wherein the L-amino acid is selected from the group consisting of L-threonine, L-lysine, L-glutamic acid, L-homoserine, L-methionine, L-arginine, L-proline and L-isoleucine.

[0014] The present invention also provides a method for producing L-amino acid comprising cultivating the above bacterium in a medium, producing and accumulating the L-amino acid in the medium, and collecting the L-amino acid 55 from the medium.

[0015] The present invention further provides the method described above wherein the L-amino acid is selected the group consisting of L-threonine, L-lysine, L-glutamic acid, L-homoserine, L-methionine, L-arginine, L-proline and L-isoleucine.

[0016] The term "L-amino acid productivity" means a property that a bacterium produces and accumulates the L-amino acid in a medium in a larger amount than a wild type strain thereof.

[0017] According to the present invention, productivity of L-amino acid such as L-threonine, L-lysine, L-glutamic acid,

L-homoserine, L-methionine, L-arginine, L-proline or L-isoleucine can be improved in a bacterium belonging to the genus *Escherichia*. Further, the present invention may be utilized to breeding of an L-amino acid producer belonging to the genus *Escherichia*.

Brief Description of the Drawings

[0018]

Figure 1 shows a scheme of constructing *Bacillus subtilis* pycA::KmR recipient strain for cloning pycA gene,

Figure 2 shows a scheme of cloning pycA gene from *Bacillus subtilis* 168, and

Figure 3 shows a scheme of construction of the plasmid containing pycA gene.

Detailed Description of the Invention

[0019] The present invention will be explained in detail below.

(1) A bacterium belonging to the genus *Escherichia* transformed with pyc gene

[0020] A bacterium belonging to the genus *Escherichia* of the present invention is a bacterium transformed with pyc gene. As the bacterium belonging to the genus *Escherichia*, there exemplified by *Escherichia coli*. While the pyc gene used in the present invention is not particularly limited so long as it can code a protein having pyruvate carboxylase activity, examples of the pyc gene include, for example, pyc gene derived from bacteria belonging to the genus *Bacillus* or pyc gene from coryneform bacteria. The nucleotide sequences of the pyc gene derived from *Bacillus subtilis* (Genbank/EMBL/DDBJ Accession Z97025, NID g2224758) and *Corynebacterium glutamicum* (Peters-Wendisch, P. G. et al., Microbiology, 144, 915-927 (1998)) has been reported. Therefore, pyc gene can be obtained by PCR (polymerase chain reaction: White, T.J. et al., Trends Genet., 5, 185 (1989)) using primers synthesized according to the nucleotide sequences from chromosomal DNA of bacteria belonging to the genus *Bacillus* such as *Bacillus subtilis* or coryneform bacteria such as *Corynebacterium glutamicum* as a template.

[0021] A transformant of *Escherichia coli* 44/pMW119-pycA, which harbors pMW119-pycA containing pyc gene of *Bacillus subtilis* has been deposited in Russian National Collection of Industrial Microorganisms (VKPM) Depository, GNIIGenetika; 1, Dorozhny Proezd., 1, 113545, Moscow, Russia, since August 30, 1999 under a Registration number of VKPM B-7822, and transferred from the original deposit to international deposit based on Budapest Treaty on September 1, 2000.

[0022] A transformant of *Escherichia coli* MG442/pMW119-pycA, which harbors pMW119-pycA containing pyc gene of *Bacillus subtilis* has been deposited in Russian National Collection of Industrial Microorganisms (VKPM) Depository, GNIIGenetika; 1, Dorozhny Proezd., 1, 113545, Moscow, Russia, since August 30, 1999 under a Registration number of VKPM B-7821, and transferred from the original deposit to international deposit based on Budapest Treaty on September 1, 2000.

[0023] Incidentally, the gene coding pyruvate carboxylase of *Bacillus subtilis* (pycA gene) has been cloned as described below in a process of accomplishing the present invention.

[0024] First, a pycA gene-deficient strain of *Bacillus subtilis* was constructed. Then, using the strain as a recipient, pycA gene was cloned by isolating a DNA fragment which complemented citrate or aspartate auxotrophy of the strain from a genomic DNA library of a wild type strain of *Bacillus subtilis*. Since *Bacillus subtilis* forms oxaloacetate by pyruvate carboxylase, *Bacillus subtilis* cannot grow on minimal medium if it is deficient in the enzyme. However the growth is repaired by addition of L-aspartate, L-glutamate, citrate or succinate. The nucleotide sequence of pycA gene and the amino acid sequence coded by the gene are shown in SEQ ID Nos: 3 and 4.

[0025] The pycA gene-deficient strain is obtained, for example, as described below. A partial DNA fragment of ylaP gene located immediately after the pycA gene on the chromosome is obtained by PCR. As primers for PCR are exemplified by oligonucleotides of SEQ ID NOs: 1 and 2. Then a wild type strain of *Bacillus subtilis* is transformed with a plasmid DNA containing the obtained DNA fragment and a marker gene, followed by integrating the plasmid DNA into ylaP gene on the chromosomal DNA by homologous recombination. Next, chromosomal DNA library of the obtained strain is constructed using a restriction enzyme which recognizes a restriction site inside of pycA gene, for example, *Pst*I. The recombinant plasmid containing the marker gene and a partial pycA gene fragment may be obtained by transforming a wild type strain of *Bacillus subtilis* with the library and selecting clones expressing the marker gene. Then a wild type

strain of *Bacillus subtilis* is transformed with the linearized recombinant plasmid and clones expressing the marker gene are selected to obtain *pycA* gene-deficient strain in which the plasmid DNA is integrated in *pycA* gene on chromosomal DNA of the strain.

[0026] Transformation of a bacterium belonging to the genus *Escherichia* with *pyc* gene may be performed by inserting *pyc* gene into an appropriate vector which functions in a bacterium belonging to the genus *Escherichia* to construct a recombinant vector, and introducing the recombinant vector to a bacterium belonging to the genus *Escherichia*.

[0027] The vector is exemplified by plasmid such as pBR322, pMW118, pMW119, pUC18, pUC19 or the like, phage vector such as λ 1059, λ BF101, M13mp9 or the like, and transposon such as Mu, Tn10, Tn5 or the like. However, in the present invention, a low copy plasmid such as pMW118 and pMW119 is preferable, because a transformant harboring a vector may be unstable if a high copy plasmid is used to introduce *pycA* gene as a vector.

[0028] The introduction of a DNA into a bacterium belonging to the genus *Escherichia* can be performed, for example, by a method of D. A. Morrison (Methods in Enzymology 68, 326 (1979)) or a method in which recipient bacterial cells are treated with calcium chloride to increase permeability of DNA (Mandel, M. and Higa, A., J. Mol. Biol., 53, 159 (1970)) and the like.

[0029] Preparation of the genomic DNA, construction of genomic DNA library, hybridization, PCR, preparation of plasmid DNA, digestion and ligation of DNA, transformation and the like are performed according to the methods which are known to a person skilled in the art. Such methods were described by Sambrook, J., Fritsche, E. F., Maniatis, T. in Molecular Cloning, Cold Spring Harbor Laboratory Press, 1.21 (1989).

[0030] L-amino acid productivity of a bacterium belonging to the genus *Escherichia* can be improved by conferring or enhancing pyruvate carboxylase activity on the bacterium through transforming the bacterium with *pyc* gene. This is probably because oxaloacetic acid is formed not only from phosphoenolpyruvic acid but also from pyruvic acid. That is, transport of glucose (or sucrose) molecule to a bacterial cell which is mediated by phosphoenolpyruvic acid system proceeds by consuming one molecule of phosphoenolpyruvic acid being accompanied with formation of one molecule of pyruvic acid. Although formed pyruvic acid is not directly utilized for L-amino acid synthesis in a bacterial cell, L-amino acid productivity may be increased by formation of oxaloacetic acid from pyruvic acid.

[0031] As a bacterium belonging to the genus *Escherichia* to which *pyc* gene is to be introduced, a strain having an ability to produce objective L-amino acid is used. Alternatively, a bacterium belonging to the genus *Escherichia* which is transformed with *pyc* gene may be conferred L-amino acid productivity. Incidentally, *pyc* gene of *Escherichia coli* has not been known, however, if the gene is found in *E. coli* hereafter, the gene may be used.

[0032] Examples of L-amino acid-producing bacteria belonging to the genus *Escherichia* are described below.

(1) L-threonine-producing bacteria

[0033] The L-threonine-producing bacteria belonging to the genus *Escherichia* may be exemplified by strain MG442 (deposited in Russian National Collection of Industrial Microorganisms (VKPM) Depositary, GNIIGenetika; 1, Dorozhny Proezd., 1, 113545, Moscow, Russia, under a Registration number of VKPM B-1628)(USP 4,278,765; Guayatinier M.M. et al., *Genetika* (in Russian), 14, 947-956 (1978)). The strain MG442 had been obtained as a mutant resistant to L-threonine analogue, 2-amino-3-hydroxyvaleric acid, it was also an isoleucine auxotroph of leaky type. It produces L-threonine and L-glutamate as a by-product.

[0034] Alternatively, a transformant of MG442 which is transformed with pVIC40 (USP 5,175,107) may be preferably used as L-threonine-producing strain.

(2) L-lysine-producing bacteria

[0035] The L-lysine-producing bacteria belonging to the genus *Escherichia* may be exemplified by various bacterium described in WO95/16042. The L-lysine-producing bacteria are concretely exemplified by a bacterium in which aspartokinase activity and dihydrodipicolinate reductase activity are increased such as W3110(*tyrA*)RSFD80+pdapB.

(3) L-homoserine-producing bacteria

[0036] The L-homoserine producing bacteria belonging to the genus *Escherichia* may be exemplified by strain 44 (*thrB*). This strain was derived from the known strain C600 (*thrB*, *leuB*) (Appleyard R.K., Genetics, 39, 440-452 (1954)) as *Leu*⁺ revertant. A transformant strain of 44 being transformed with a plasmid containing a *thrA* gene coding for aspartokinase-homoserine dehydrogenase I may be preferably used.

[0037] A strain of *Escherichia coli* 44 has been deposited in Russian National Collection of Industrial Microorganisms (VKPM) Depositary, GNIIGenetika; 1, Dorozhny Proezd., 1, 113545, Moscow, Russia, since September 23, 1980 under a Registration number of VKPM B-2175, and transferred from the original deposit to international deposit based on Budapest Treaty on September 1, 2000.

(4) L-glutamic acid-producing bacteria

[0038] The L-glutamic acid producing bacteria belonging to the genus *Escherichia* may be exemplified by a strain which is resistant to L-valine, for example, *Escherichia coli* strains B11, K-12 (ATCC10798), B (ATCC11303) and W (ATCC9637).

(5) L-isoleucine-producing bacteria

[0039] The L-isoleucine-producing bacteria belonging to the genus *Escherichia* may be exemplified by a strain TDH6/pVIC40, pMWD5 (Hashiguchi, K. et al., Biosci. Biotechnol. Biochem., 63(4), 672-679 (1999)) or AJ12919 described in EP0 685 555 A1.

(2) Method for producing an L-amino acid

[0040] An L-amino acid can be efficiently produced by cultivating a bacterium belonging to the genus *Escherichia* which is transformed with *pycA* gene as described above and has an L-amino acid productivity in an appropriate medium, producing and accumulating the L-amino acid in the medium, and collecting the L-amino acid from the medium.

[0041] The L-amino acid is exemplified preferably by L-threonine, L-lysine, L-glutamic acid, L-homoserine, L-methionine, L-arginine, L-proline and L-isoleucine. The L-amino acid may be produced by the method of the present invention either alone or as a mixture of more than two kinds of L-amino acids.

[0042] In the method of present invention, the cultivation of the bacterium belonging to the genus *Escherichia*, the collection and purification of amino acid from the liquid medium may be performed in a manner similar to those of the conventional method for producing an amino acid by fermentation using a bacterium. A medium used in cultivation may be either a synthetic medium or a natural medium, so long as the medium includes a carbon and a nitrogen source and minerals and, if necessary, nutrients which the bacterium used requires for growth in appropriate amounts. The carbon source may include various carbohydrates such as glucose and sucrose, and various organic acids. Depending on assimilatory ability of the used bacterium, alcohol including ethanol and glycerol may be used. As the nitrogen source, ammonia, various ammonium salts such as ammonium sulfate, other nitrogen compounds such as amines, a natural nitrogen source such as peptone, soybean hydrolyzate and digested fermentative microbe are used. As minerals, monopotassium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, calcium carbonate are used.

[0043] The cultivation is preferably performed under an aerobic condition such as a shaking culture, and an aeration and stirring culture. The temperature of culture is usually 20 to 40°C, preferably 30 to 38°C. The pH of the culture is usually between 5 and 9, preferably between 6.5 and 7.2. The pH of the culture can be adjusted with ammonia, calcium carbonate, various acids, various bases, and buffers. Usually, 1 to 3-day cultivation leads to the accumulation of the target L-amino acid in the medium.

[0044] Collecting and purifying the L-amino acid can be performed by removing solids such as cells from the medium by centrifugation or membrane filtration after cultivation, followed by ion exchange, concentration and crystalline fraction methods and the like.

Best Mode for Carrying Out the Invention

[0045] The present invention will be more concretely explained below with reference to Examples.

Example 1: Cloning of the *pycA* gene from *Bacillus subtilis* 168 strain(1) Construction of *B. subtilis* *pycA::KmR* recipient strain for cloning

[0046] The recipient strain of *B. subtilis* was constructed by insertional mutagenesis of *pycA* gene. The fragment of *pycA* gene was cloned in two steps (refer to Figs. 1 and 2).

[0047] In a data bank one of reading frame (ylaP) from *B. subtilis* genome sequence project is identified as *pycA* gene, encoding pyruvate carboxylase. The region located immediately after the *pycA* gene terminator was cloned by PCR amplification, using the following pair of oligonucleotides:

5' -GATATAAGGGGACTTCAGAG, and
5' -GGCGCTTTATGCGTTTCAATC.

[0048] The 269bp DNA fragment was blunt-ended with Klenow fragment of DNA polymerase I and cloned into *ScaI* site of pBR322 plasmid in *E. coli* strain. The resulting plasmid pBRPYCA11 was digested by *ClaI* and ligated to 1628bp DNA fragment carrying *cat*-gene, which was obtained by digestion of pC194 with *ClaI*. Then, using the obtained plasmid pBRPYCA11CmR3, *Bacillus subtilis* 168 trpC2 strain was transformed and Cm^R-clones were selected. The plasmid pBRPYCA11CmR3 cannot autonomously replicate in bacterium belonging to the genus *Bacillus*, but can integrate into chromosome near the *pycA* gene by homology with the 269bp DNA fragment. The strain was named as *B. subtilis* trpC2 xyz::pBRPYCACmR3. The integrant carried pBR322-replicon, *tet* gene encoding resistance to tetracycline derived from pBR322 and Cm^R marker.

[0049] Chromosomal DNA of *B. subtilis* trpC2 xyz::pBRPYCACmR3 was isolated and used to clone a distal part of *pycA* gene. The chromosomal DNA which is digested with *PstI* was self-ligated and transformed into *E. coli* TG1 strain. Recombinants resistant to tetracycline and chloramphenicol were selected. The plasmid was recovered from one of the recombinant and named as pPYC1. The plasmid pPYC1 contained a single *BglII* restriction site, which located in *pycA* gene fragment. This site was used to insert 1818bp DNA fragment, containing a kanamycin resistance marker for *B. subtilis*. The plasmid pPYC1 was digested with *BglII* and ligated to pUC7KmR which was digested with *BamHI* to obtain a plasmid pPYC1::KmR.

[0050] The inactivation of *pycA* gene in a wild type *B. subtilis* 168 trpC2 was achieved by transformation of the same strain with linearized plasmid pPYC1::KmR by one step procedure, followed by selection on Luria broth supplemented with kanamycin. The Km^R marker was inserted into *pycA* gene on chromosomal DNA by homologous double-crossover recombination.

[0051] The constructed strain of *B. subtilis* *pycA*::Km^R trpC2 did not grow in Spizizen minimal medium (Anagnostopoulos, C and Spizizen, J., J. Bacteriol., 81, 741-746 (1961)) with tryptophan unless citrate or aspartate were added to the media.

(Composition of Spizizen minimal medium)

[0052]

K ₂ HPO ₄ • 3H ₂ O	18.3 g/L
KH ₂ PO ₄	6.0 g/L
(NH ₄) ₂ SO ₄	2.0 g/L
sodium citrate • 5H ₂ O	1.2 g/L
MgSO ₄ • 7H ₂ O	0.2 g/L
Glucose	10.0 g/L
pH7.0	

[0053] The *B. subtilis* trpC2 *pycA*::Km^R *recE*::Tc^R strain was constructed by transducing the inactivated allele of *recE* gene from *B. subtilis* *recE*::Tc^R strain with phage E40 (Fig. 2). It was necessary to inactivate recombination in recipient strain for cloning of *pycA* gene from *B. subtilis*. The constructed strain *B. subtilis* trpC2 *pycA*::Km^R *recE*::Tc^R strain was used as recipient to clone *pycA* gene.

(2) Cloning of *B. subtilis* native *pycA* gene by complementation.

[0054] The *pycA* gene was cloned from *B. subtilis* 168 trpC2 strain. Chromosomal DNA prepared from the strain 168 trpC2 was partially digested with *EcoRI* and ligated to pCB20 (Em^R) (gifted by Dr. Yu Jomantas, refer to "Genetic transformation and expression" L.O. Butler, et al. eds., Intercept Ltd., PO Box 402, Wimborne, Dorset, BH229T2, U.K., 1989, p269-281) which was digested with *EcoRI*. Then the trpC2 *pycA*::Km^R *recE*::Tc^R recipient strain was transformed with the ligation mixture (Fig. 2). The Asp⁺, Em^R, Km^R clones were selected on Spizizen minimal medium without citrate or aspartate supplementation but supplemented with tryptophan, erythromycin and kanamycin. The plasmid pPYCR3 harbored by one of the selected clones complemented *pycA* mutation, had a predicted structure and conferred the same Asp⁺, Em^R phenotype after second transformation.

(3) Cloning of DNA fragment carrying *pycA* gene from *B. subtilis* 168 trpC2 strain into high copy number plasmid pUC18 and pUC19

[0055] Plasmid pPYCR3 was digested with *Hind*II and *Sca*I and *Hind*II-*Sca*I fragment (4414 bp), carrying *pycA* gene with 5'-end upstream sequence (including promoter and ribosome binding site), was cloned in pUC18 digested with *Sma*I. Thus the plasmid pUC18-*pycA* was obtained (Fig. 3, upside). The ligation reaction was performed concretely as follows. Ninety ng of *Sma*I-digested pUC18 and 300 ng of *Hind*II-*Sca*I fragment (4414 bp) carrying *pycA* gene which was purified by agarose gel electrophoresis were ligated with 2 units of T4 DNA Ligase (Pharmacia, Sweden) in 45 µl of reaction mixture containing 1x One-Phor-All buffer (Pharmacia, Sweden) and 1 mM ATP.

[0056] *E. coli* TG1 was transformed with pUC18-*pycA*. Obtained transformants were analyzed by using *Kpn*I, *Xba*I, *Bam*HI, *Hind*III and *Eco*RI endonucleases. All clones carried the *pycA* gene in opposite direction to *lac* promoter of pUC18 plasmid. However, the *pycA* genes cloned in pUC18 may be expressed under the control of the inherent promoter of the gene.

[0057] Then in order to rearrange *pycA* gene under control of *lac* promoter, the *Kpn*I-*Xba*I fragment of pUC18-*pycA* was cloned into pUC19 digested with *Kpn*I-*Xba*I. However, the obtained clones were unstable and the recombinant plasmid was eliminated during short time (6-8 h) of cultivation.

(4) Cloning of DNA fragment carrying *pycA* gene into a low copy number plasmid pMW119

[0058] The *Kpn*I-*Xba*I fragment of pUC18-*pycA* was ligated to a low copy number plasmid pMW119 digested with *Kpn*I and *Xba*I (Fig. 3, downside). The ligation reaction was performed in 50 µl of reaction mixture containing 60 ng of pMW119 digested with *Kpn*I-*Xba*I, 120 ng of *Kpn*I-*Xba*I fragment of pUC18-*pycA* which was purified by agarose gel electrophoresis, 1x One-Phor-All buffer (Pharmacia, Sweden), 1 mM ATP and 1 unit of T4 DNA Ligase (Pharmacia, Sweden).

[0059] Using the ligation mixture, *E. coli* TG1 was transformed. Resulting transformants were analyzed using *Sac*I, *Kpn*I, *Xba*I, *Bam*HI, *Hind*III and *Eco*RI. All clones carried *pycA* gene under control of *lac* promoter of pMW119 and its own promoter. The thus obtained clone was named TG1(pMW119-*pycA*), from which the plasmid pMW119-*pycA* was obtained.

Example 2: Production of L-threonine and L-glutamic acid

[0060] *E. coli* strain MG442 was transformed with the plasmid pMW119-*pycA* and 10 Amp^r colonies were selected. Productivities of L-threonine and L-glutamic acid of the colonies were examined using the following fermentation medium.

(Composition of fermentation medium)

[0061]

glucose (separately sterilized)	60 g/L
(NH ₄) ₂ SO ₄	15.0 g/L
KH ₂ PO ₄	1.5 g/L
MgSO ₄ · 7H ₂ O	1.0 g/L
L-isoleucine	100 mg/L
thiamine	0.1 mg/L
CaCO ₃ (separately sterilized)	20 g/L

[0062] Fermentation was performed in test tubes (20x300mm) at 32 °C for 72 hours using a rotary shaker. One loop of each of 10 clones was inoculated into the test tube containing 2.0 ml of the fermentation medium. As controls, 10 single colonies of a plasmid-less strain MG442 were used. After the fermentation, concentrations of L-threonine and L-glutamic acid accumulated in the media were measured by thin-layer chromatography. The results are shown in Table 1.

Table 1

Clone Nos.	MG442(control)		MG442(pMW119-pycA)	
	L-thr G/L	L-glu G/L	L-thr g/L	L-glu g/L
1	5.5	9.5	7.8	12.3
2	3.2	7.1	9.8	12.3
3	3.6	7.1	9.4	13.3
4	3.4	8.9	9.8	11.8
5	3.3	8.9	9.8	11.8
6	4.8	7.3	9.9	11.8
7	3.3	7.0	10.0	10.7
8	4.7	8.3	10.0	10.4
9	3.2	7.3	9.7	11.2
10	2.6	4.1	10.0	10.7
Average	3.8	7.4	9.6	11.6

Example 3: Production of L-homoserine

[0063] L-homoserine-producing E. coli strain 44 was transformed with the plasmid pMW119-pycA and five ampiciline resistant clones were selected. Production of L-homoserine was examined as it is indicated in the Example 1, but the fermentation medium was supplemented with 0.5 g/l of L-threonine. As a control five colonies of plasmidless strain 44 were used. After the fermentation average L-homoserine concentrations were 4.5 g/l (the strain 44) and 5.7 g/l (the strain 44 with the plasmid pMW119-pycA).

[0064] Also the effect of pycA gene on the productivity of L-lysine is reasonably expected, because pycA gene is confirmed to have the positive effect on the productivity of L-threonine and L-homoserine which share the same biosynthetic pathway in part as L-lysine.

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5

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10

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<213> Artificial Sequence

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<212> DNA

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<220>

<221> CDS

<222> (16)..(3459)

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aac agg gga gaa att gca atc cga ata ttc cgg gcg tgt acc gag ttg 99

Asn Arg Gly Glu Ile Ala Ile Arg Ile Phe Arg Ala Cys Thr Glu Leu

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aat att cgt aca gtt gcg gtc tat tca aaa gaa gat tcc ggt tcc tac 147

Asn Ile Arg Thr Val Ala Val Tyr Ser Lys Glu Asp Ser Gly Ser Tyr

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cat cgg tac aaa gcg gat gaa gca tac ttg gtc ggt gaa ggg aaa aaa 195

His Arg Tyr Lys Ala Asp Glu Ala Tyr Leu Val Gly Glu Gly Lys Lys

45

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ccg att gat gct tac ctg gat att gaa ggt atc att gat att gcg aaa 243

Pro Ile Asp Ala Tyr Leu Asp Ile Glu Gly Ile Ile Asp Ile Ala Lys

65

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aga aac aaa gtc gat gca att cat ccg gga tac ggt ttc tta tct gaa 291

Arg Asn Lys Val Asp Ala Ile His Pro Gly Tyr Gly Phe Leu Ser Glu

80

85

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aat att cat ttt gcg aga cga tgt gaa gaa gaa ggc atc gta ttc ata 339

EP 1 092 776 A1

Asn Ile His Phe Ala Arg Arg Cys Glu Glu Glu Gly Ile Val Phe Ile
 95 100 105
 5 ggg cca aaa tcc gag cat ctc gat atg ttt ggt gac aag gta aaa gcg 387
 Gly Pro Lys Ser Glu His Leu Asp Met Phe Gly Asp Lys Val Lys Ala
 110 115 120
 10 cgt gag cag gca gaa aaa gcg gga atc ccc gtg att ccg gga agc gac 435
 Arg Glu Gln Ala Glu Lys Ala Gly Ile Pro Val Ile Pro Gly Ser Asp
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 15 ggt cct gcc gaa acg ctt gaa gcc gtc gaa caa ttt gga caa gct aac 483
 Gly Pro Ala Glu Thr Leu Glu Ala Val Glu Gln Phe Gly Gln Ala Asn
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 20 ggt tat ccg atc atc att aaa gcc tcg ctt ggc ggc ggc ggc cgc ggt 531
 Gly Tyr Pro Ile Ile Ile Lys Ala Ser Leu Gly Gly Gly Gly Arg Gly
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 25 atg cgg att gtc aga tct gaa agt gaa gtt aaa gaa gca tat gag cgt 579
 Met Arg Ile Val Arg Ser Glu Ser Glu Val Lys Glu Ala Tyr Glu Arg
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 30 gct aaa tca gag gcg aaa gca gcc ttt ggc aat gat gaa gtt tat gta 627
 Ala Lys Ser Glu Ala Lys Ala Ala Phe Gly Asn Asp Glu Val Tyr Val
 190 195 200
 35 gaa aaa tta att gag aat ccg aaa cat att gag gtt cag gtc att gga 675
 Glu Lys Leu Ile Glu Asn Pro Lys His Ile Glu Val Gln Val Ile Gly
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 40 gac aag cag ggc aat gtc gtc cat ctt ttt gag agg gat tgc tcc gtt 723
 Asp Lys Gln Gly Asn Val Val His Leu Phe Glu Arg Asp Cys Ser Val
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 45 caa aga cgc cat caa aaa gtc att gaa gtg gcg ccg agt gtc tcg ctg 771
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 50 tca cct gaa tta agg gac caa att tgt gag gct gca gtt gcg ctt gcc 819
 Ser Pro Glu Leu Arg Asp Gln Ile Cys Glu Ala Ala Val Ala Leu Ala
 255 260 265
 55 aaa aat gta aac tat ata aat gcg ggg acg gtc gaa ttc ctt gtt gca 867
 Lys Asn Val Asn Tyr Ile Asn Ala Gly Thr Val Glu Phe Leu Val Ala

EP 1 092 776 A1

	270	275	280	
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5	Asn Asn Glu Phe Tyr Phe Ile Glu Val Asn Pro Arg Val Gln Val Glu			
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	atc ctt gtt gcc caa ggg cac agc ctt cac agc aaa aaa gta aat att			1011
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20	Pro Glu Gln Lys Asp Ile Phe Thr Ile Gly Tyr Ala Ile Gln Ser Arg			
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	gtt acg act gag gat cgc caa aat gat ttc atg cct gat aca gga aaa			1107
25	Val Thr Thr Glu Asp Pro Gln Asn Asp Phe Met Pro Asp Thr Gly Lys			
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30	Ile Met Ala Tyr Arg Ser Gly Gly Gly Phe Gly Val Arg Leu Asp Thr			
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	gga aac agc ttc cag ggc gcc gtg atc aca cca tac tat gat tca ctt			1203
	Gly Asn Ser Phe Gln Gly Ala Val Ile Thr Pro Tyr Tyr Asp Ser Leu			
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	Asn Ile Pro Phe Leu Glu Asn Val Ala Lys His Glu Lys Phe Leu Thr			
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50	ggg caa tat gat aca tct ttc att gat aca acg cct gaa tta ttt aat			1395
	Gly Gln Tyr Asp Thr Ser Phe Ile Asp Thr Thr Pro Glu Leu Phe Asn			
	445	450	455	460

EP 1 092 776 A1

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	Ala Phe Asp Lys Pro Leu Gly Val Lys Val Asp Val Asp Gln Gln Pro	
	495 500 505	
15	gcc aga gga aca aag caa att ctc gat gaa aaa ggt gca gaa ggg ctt	1587
	Ala Arg Gly Thr Lys Gln Ile Leu Asp Glu Lys Gly Ala Glu Gly Leu	
	510 515 520	
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25	ttc agg gat gcc cac caa tcg tta ttg gca act aga atc aga tcg cat	1683
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	Asp Leu Lys Lys Ile Ala Asn Pro Thr Ala Ala Leu Trp Pro Glu Leu	
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35	ttc agt atg gaa atg tgg gga ggc gcg acc ttc gat gta gcc tac cga	1779
	Phe Ser Met Glu Met Trp Gly Gly Ala Thr Phe Asp Val Ala Tyr Arg	
	575 580 585	
40	ttc ctg aaa gaa gat ccg tgg aaa cgt ttg gaa gat ctt cgc aaa gaa	1827
	Phe Leu Lys Glu Asp Pro Trp Lys Arg Leu Glu Asp Leu Arg Lys Glu	
	590 595 600	
45	gtg ccg aat acc tta ttc cag atg ttg ctt cgc tca tca aat gcg gtc	1875
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	605 610 615 620	
50	ggc tat acg aat tat ccg gac aat gtg att aaa gaa ttt gtg aag caa	1923
	Gly Tyr Thr Asn Tyr Pro Asp Asn Val Ile Lys Glu Phe Val Lys Gln	
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55	tca gct caa tcc ggt att gat gtg ttt cgt att ttc gac agc tta aac	1971

EP 1 092 776 A1

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5	tgg gta aaa ggg atg acg tta gcc att gat gct gtt agg gat acc ggc	2019
	Trp Val Lys Gly Met Thr Leu Ala Ile Asp Ala Val Arg Asp Thr Gly	
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10	aaa gtg gca gaa gct gcg att tgt tat acg gga gat atc ctt gac aag	2067
	Lys Val Ala Glu Ala Ala Ile Cys Tyr Thr Gly Asp Ile Leu Asp Lys	
	670 675 680	
15	aac cgg acg aag tac gac ctt gca tat tat aca tcg atg gcg aag gag	2115
	Asn Arg Thr Lys Tyr Asp Leu Ala Tyr Tyr Thr Ser Met Ala Lys Glu	
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	Leu Glu Ala Ala Gly Ala His Ile Leu Gly Ile Lys Asp Met Ala Gly	
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25	ctg tta aaa ccg cag gct gca tat gag ctc gtt tct gcg ttg aaa gaa	2211
	Leu Leu Lys Pro Gln Ala Ala Tyr Glu Leu Val Ser Ala Leu Lys Glu	
	720 725 730	
30	acg atc gac att ccg gtt cac ctt cat acg cat gat acg agc gga aac	2259
	Thr Ile Asp Ile Pro Val His Leu His Thr His Asp Thr Ser Gly Asn	
	735 740 745	
35	ggg att tat atg tat gcg aaa gct gtt gaa gcc ggc gtt gat atc ata	2307
	Gly Ile Tyr Met Tyr Ala Lys Ala Val Glu Ala Gly Val Asp Ile Ile	
	750 755 760	
40	gac gtg gcg gtc agc tca atg gcg gga tta acg tca cag cct agc gcg	2355
	Asp Val Ala Val Ser Ser Met Ala Gly Leu Thr Ser Gln Pro Ser Ala	
	765 770 775 780	
45	agc gga ttt tat cat gcg atg gaa ggc aac gac cgc cgt ccg gaa atg	2403
	Ser Gly Phe Tyr His Ala Met Glu Gly Asn Asp Arg Arg Pro Glu Met	
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50	aat gtc caa ggc gtt gaa ttg ctg tcc caa tat tgg gag tgc gtg cgt	2451
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	800 805 810	
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EP 1 092 776 A1

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	845	850	855	860
	atg tac aga cgc gtg aac gat atg ttc ggt gac atc gtc aag gta acg	2643		
15	Met Tyr Arg Arg Val Asn Asp Met Phe Gly Asp Ile Val Lys Val Thr			
	865	870	875	
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20	Pro Ser Ser Lys Val Val Gly Asp Met Ala Leu Tyr Met Val Gln Asn			
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35	ccg att aca gtc aga ccg ggc gaa ctg ctt gag ccg gtg tca ttt gaa	2883		
	Pro Ile Thr Val Arg Pro Gly Glu Leu Leu Glu Pro Val Ser Phe Glu			
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40	gcg atc aaa cag gaa ttt aaa gag cag cat aac ttg gaa att tca gat	2931		
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45	cag gat gct gtg gca tat gcc ctt tat cct aaa gtc ttc act gat tat	2979		
	Gln Asp Ala Val Ala Tyr Ala Leu Tyr Pro Lys Val Phe Thr Asp Tyr			
	975	980	985	
50	gtg aaa acg aca gaa agc tat gga gac atc tcg gta tta gat aca ccg	3027		
	Val Lys Thr Thr Glu Ser Tyr Gly Asp Ile Ser Val Leu Asp Thr Pro			
	990	995	1000	

EP 1 092 776 A1

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Glu Arg Gly Lys Thr Leu Ile Val Lys Leu Ile Ser Ile Gly Glu Pro
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1105 1110 1115
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1120 1125 1130
ggt gag ccg atc caa acg gga gat ctg ctc ctt gaa att gaa aaa gca 3459
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taa 3462

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<211> 1148

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<213> Bacillus subtilis

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 1 5 10 15
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 10 Val Ala Val Tyr Ser Lys Glu Asp Ser Gly Ser Tyr His Arg Tyr Lys
 35 40 45
 Ala Asp Glu Ala Tyr Leu Val Gly Glu Gly Lys Lys Pro Ile Asp Ala
 50 55 60
 15 Tyr Leu Asp Ile Glu Gly Ile Ile Asp Ile Ala Lys Arg Asn Lys Val
 65 70 75 80
 Asp Ala Ile His Pro Gly Tyr Gly Phe Leu Ser Glu Asn Ile His Phe
 20 85 90 95
 Ala Arg Arg Cys Glu Glu Glu Gly Ile Val Phe Ile Gly Pro Lys Ser
 100 105 110
 25 Glu His Leu Asp Met Phe Gly Asp Lys Val Lys Ala Arg Glu Gln Ala
 115 120 125
 Glu Lys Ala Gly Ile Pro Val Ile Pro Gly Ser Asp Gly Pro Ala Glu
 130 135 140
 30 Thr Leu Glu Ala Val Glu Gln Phe Gly Gln Ala Asn Gly Tyr Pro Ile
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 Ile Ile Lys Ala Ser Leu Gly Gly Gly Gly Arg Gly Met Arg Ile Val
 35 165 170 175
 Arg Ser Glu Ser Glu Val Lys Glu Ala Tyr Glu Arg Ala Lys Ser Glu
 180 185 190
 40 Ala Lys Ala Ala Phe Gly Asn Asp Glu Val Tyr Val Glu Lys Leu Ile
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 Glu Asn Pro Lys His Ile Glu Val Gln Val Ile Gly Asp Lys Gln Gly
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 45 Asn Val Val His Leu Phe Glu Arg Asp Cys Ser Val Gln Arg Arg His
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 50 245 250 255
 Arg Asp Gln Ile Cys Glu Ala Ala Val Ala Leu Ala Lys Asn Val Asn

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EP 1 092 776 A1

5 260 265 270
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 290 295 300
 10 Glu Met Ile Thr Gly Val Asp Ile Val Gln Thr Gln Ile Leu Val Ala
 305 310 315 320
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 325 330 335
 15 Asp Ile Phe Thr Ile Gly Tyr Ala Ile Gln Ser Arg Val Thr Thr Glu
 340 345 350
 Asp Pro Gln Asn Asp Phe Met Pro Asp Thr Gly Lys Ile Met Ala Tyr
 20 355 360 365
 Arg Ser Gly Gly Gly Phe Gly Val Arg Leu Asp Thr Gly Asn Ser Phe
 370 375 380
 25 Gln Gly Ala Val Ile Thr Pro Tyr Tyr Asp Ser Leu Leu Val Lys Leu
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 405 410 415
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 40 Lys Asp Arg Gly Thr Lys Met Leu Thr Tyr Ile Gly Asn Val Thr Val
 465 470 475 480
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 485 490 495
 45 Pro Leu Gly Val Lys Val Asp Val Asp Gln Gln Pro Ala Arg Gly Thr
 500 505 510
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 50 515 520 525
 Lys Glu Gln Lys Ser Val Leu Leu Thr Asp Thr Thr Phe Arg Asp Ala

EP 1 092 776 A1

	530	535	540
	His Gln Ser Leu Leu Ala Thr Arg Ile Arg Ser His Asp Leu Lys Lys		
5	545	550	555 560
	Ile Ala Asn Pro Thr Ala Ala Leu Trp Pro Glu Leu Phe Ser Met Glu		
	565	570	575
10	Met Trp Gly Gly Ala Thr Phe Asp Val Ala Tyr Arg Phe Leu Lys Glu		
	580	585	590
	Asp Pro Trp Lys Arg Leu Glu Asp Leu Arg Lys Glu Val Pro Asn Thr		
15	595	600	605
	Leu Phe Gln Met Leu Leu Arg Ser Ser Asn Ala Val Gly Tyr Thr Asn		
	610	615	620
	Tyr Pro Asp Asn Val Ile Lys Glu Phe Val Lys Gln Ser Ala Gln Ser		
20	625	630	635 640
	Gly Ile Asp Val Phe Arg Ile Phe Asp Ser Leu Asn Trp Val Lys Gly		
	645	650	655
25	Met Thr Leu Ala Ile Asp Ala Val Arg Asp Thr Gly Lys Val Ala Glu		
	660	665	670
	Ala Ala Ile Cys Tyr Thr Gly Asp Ile Leu Asp Lys Asn Arg Thr Lys		
30	675	680	685
	Tyr Asp Leu Ala Tyr Tyr Thr Ser Met Ala Lys Glu Leu Glu Ala Ala		
	690	695	700
	Gly Ala His Ile Leu Gly Ile Lys Asp Met Ala Gly Leu Leu Lys Pro		
35	705	710	715 720
	Gln Ala Ala Tyr Glu Leu Val Ser Ala Leu Lys Glu Thr Ile Asp Ile		
	725	730	735
40	Pro Val His Leu His Thr His Asp Thr Ser Gly Asn Gly Ile Tyr Met		
	740	745	750
	Tyr Ala Lys Ala Val Glu Ala Gly Val Asp Ile Ile Asp Val Ala Val		
45	755	760	765
	Ser Ser Met Ala Gly Leu Thr Ser Gln Pro Ser Ala Ser Gly Phe Tyr		
	770	775	780
	His Ala Met Glu Gly Asn Asp Arg Arg Pro Glu Met Asn Val Gln Gly		
50	785	790	795 800
	Val Glu Leu Leu Ser Gln Tyr Trp Glu Ser Val Arg Lys Tyr Tyr Ser		

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EP 1 092 776 A1

	805	810	815
	Glu Phe Glu Ser Gly Met Lys Ser Pro His Thr Glu Ile Tyr Glu His		
5	820	825	830
	Glu Met Pro Gly Gly Gln Tyr Ser Asn Leu Gln Gln Gln Ala Lys Gly		
	835	840	845
10	Val Gly Leu Gly Asp Arg Trp Asn Glu Val Lys Glu Met Tyr Arg Arg		
	850	855	860
	Val Asn Asp Met Phe Gly Asp Ile Val Lys Val Thr Pro Ser Ser Lys		
15	865	870	875
	Val Val Gly Asp Met Ala Leu Tyr Met Val Gln Asn Asn Leu Thr Glu		
	885	890	895
	Lys Asp Val Tyr Glu Lys Gly Glu Ser Leu Asp Phe Pro Asp Ser Val		
20	900	905	910
	Val Glu Leu Phe Lys Gly Asn Ile Gly Gln Pro His Gly Gly Phe Pro		
	915	920	925
25	Glu Lys Leu Gln Lys Leu Ile Leu Lys Gly Gln Glu Pro Ile Thr Val		
	930	935	940
	Arg Pro Gly Glu Leu Leu Glu Pro Val Ser Phe Glu Ala Ile Lys Gln		
30	945	950	955
	Glu Phe Lys Glu Gln His Asn Leu Glu Ile Ser Asp Gln Asp Ala Val		
	965	970	975
	Ala Tyr Ala Leu Tyr Pro Lys Val Phe Thr Asp Tyr Val Lys Thr Thr		
35	980	985	990
	Glu Ser Tyr Gly Asp Ile Ser Val Leu Asp Thr Pro Thr Phe Phe Tyr		
	995	1000	1005
40	Gly Met Thr Leu Gly Glu Glu Ile Glu Val Glu Ile Glu Arg Gly Lys		
	1010	1015	1020
	Thr Leu Ile Val Lys Leu Ile Ser Ile Gly Glu Pro Gln Pro Asp Ala		
45	1025	1030	1035
	Thr Arg Val Val Tyr Phe Glu Leu Asn Gly Gln Pro Arg Glu Val Val		
	1045	1050	1055
	Ile Lys Asp Glu Ser Ile Lys Ser Ser Val Gln Glu Arg Leu Lys Ala		
50	1060	1065	1070
	Asp Arg Thr Asn Pro Ser His Ile Ala Ala Ser Met Pro Gly Thr Val		

55

1075 1080 1085
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 5 1090 1095 1100
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 105 1110 1115 1120
 10 Phe Ser Gly Thr Ile Lys Gln Val His Val Lys Asn Gly Glu Pro Ile
 1125 1130 1135
 Gln Thr Gly Asp Leu Leu Leu Glu Ile Glu Lys Ala
 15 1140 1145

Claims

1. A bacterium belonging to the genus Escherichia which contains a gene coding for an enzyme having pyruvate carboxylase activity and which has L-amino acid productivity.
2. The bacterium according to claim 1, wherein the gene coding for an enzyme having pyruvate carboxylase activity is pyruvate carboxylase derived from a bacterium belonging to the genus Bacillus.
3. The bacterium according to claim 1 or 2, wherein said gene has the nucleotide sequence as shown in SEQ ID NO 3.
4. The bacterium according to any of the preceding claims, wherein said enzyme has an amino acid sequence as shown in SEQ ID NO 4.
5. The bacterium according to any of the preceding claims which is transformed with a vector containing said gene.
6. The bacterium according to any of the preceding claims, wherein the gene is introduced into said bacterium in a low copy number.
7. The bacterium according to any of the preceding claims, wherein the L-amino acid is selected from the group consisting of L-threonine, L-lysine, L-glutamic acid, L-homoserine, L-methionine, L-arginine, L-proline and L-isoleucine.
8. The bacterium according to any of the preceding claims which produces a higher amount of the L-amino acid than the bacterium not containing said gene.
9. The bacterium according to any of the preceding claims which produces L-threonine in a concentration which is at least 100 % higher than the concentration of L-threonine produced by a bacterium not containing said gene.
10. The bacterium according to any of the preceding claims which produces L-glutamic acid in a concentration which is at least 50 % higher than the concentration of L-glutamic acid produced by a bacterium not containing said gene.
11. The bacterium according to any of the preceding claims which produces L-homoserine in a concentration which is at least 25 % higher than the concentration of L-homoserine produced by a bacterium not containing said gene.
12. The bacterium having the accession number VKPM B-7821.
13. The bacterium having the accession number VKPM B-7822.

EP 1 092 776 A1

14. A method for producing an L-amino acid comprising cultivating the bacterium according to any of the claims 1 to 13 in a medium, producing and accumulating the L-amino acid in the medium.

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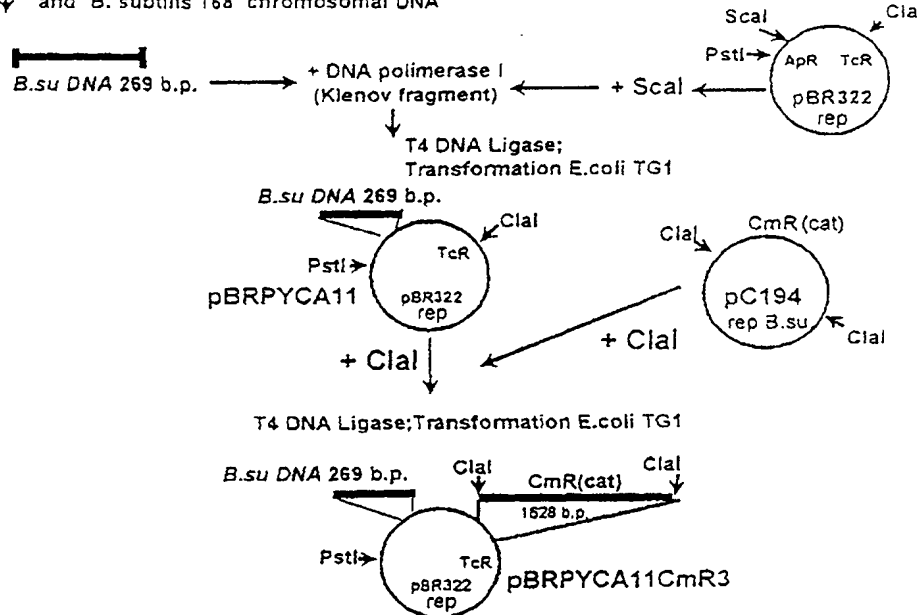
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PCR with oligonucleotides: 5'-GATATAAGGGGACTTCAGAG and 5'-GGCGCTTTATGCGTTTCAATC
↓ and *B. subtilis* 168 chromosomal DNA



Transformation of *B. subtilis* 168 trpC2; and selection of CmR-clone of integrant
B. subtilis trpC2 xyz(269 b.p.):pBRPYCA11CmR3;

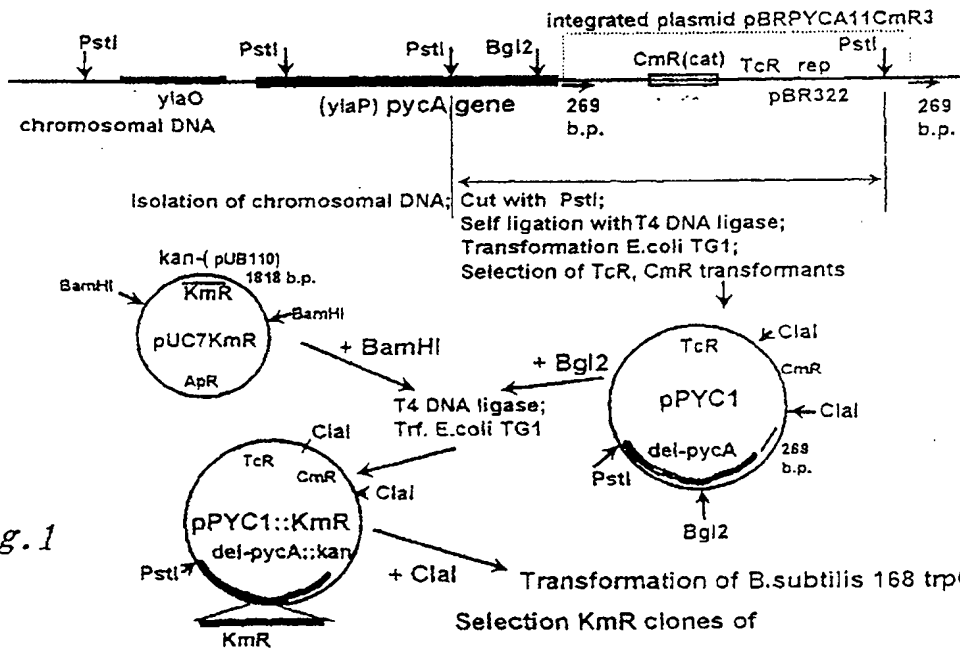


Fig.1

B. subtilis trpC2 pycA::KmR (phenotype Asp(-) Trp(-) KmR)

B. subtilis trpC2 pycA::KmR (phenotype Asp(-) KmR)
 ↓
 Transduction with phages E40 lysate prepared on
B. subtilis recE::tet (TcR) strain;
 Selection of TcR clones of
B. subtilis trpC2 pycA::KmR recE::TcR
 (phenotype Asp(-) Trp(-) KmR TcR)
 This strain was used as the recipient for the cloning of pycA gene.

Chromosomal DNA of *B. subtilis* 168 trpC2 was partially digested with EcoRI.

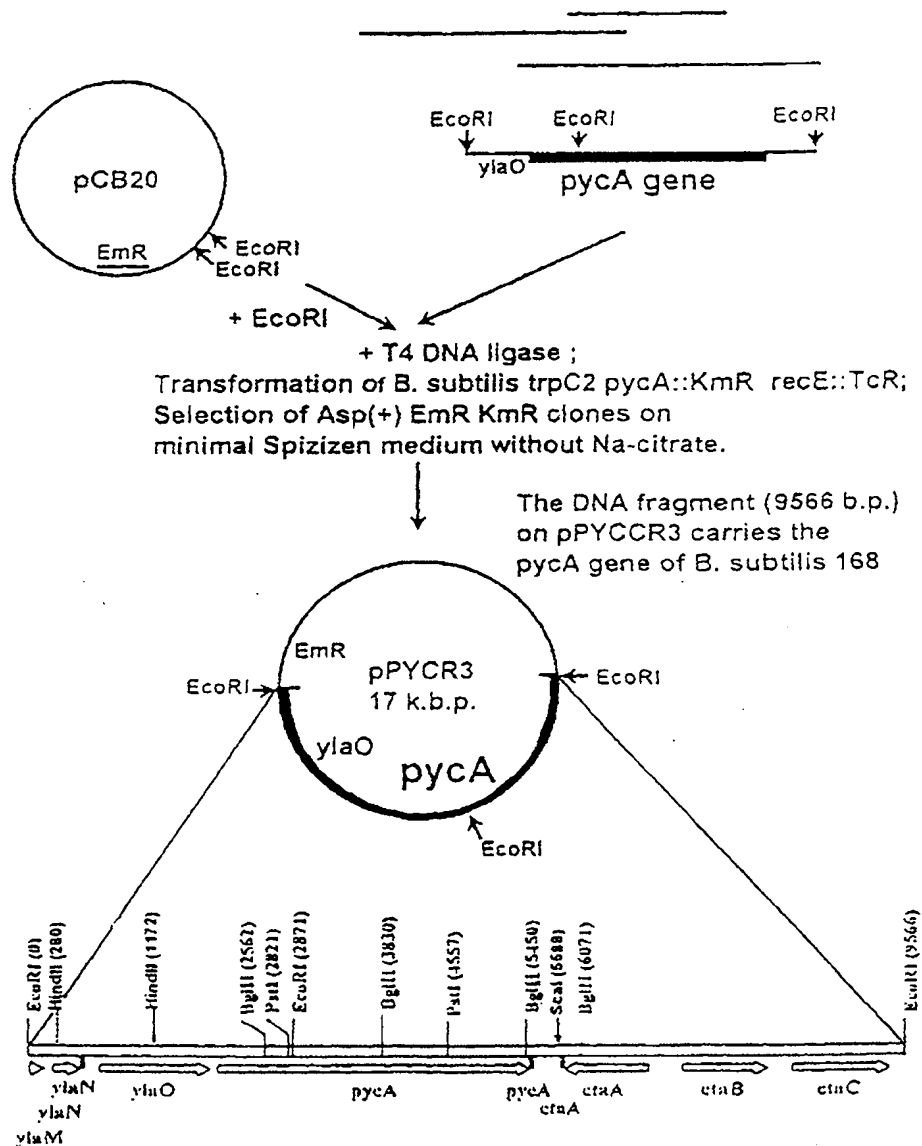


Fig.2

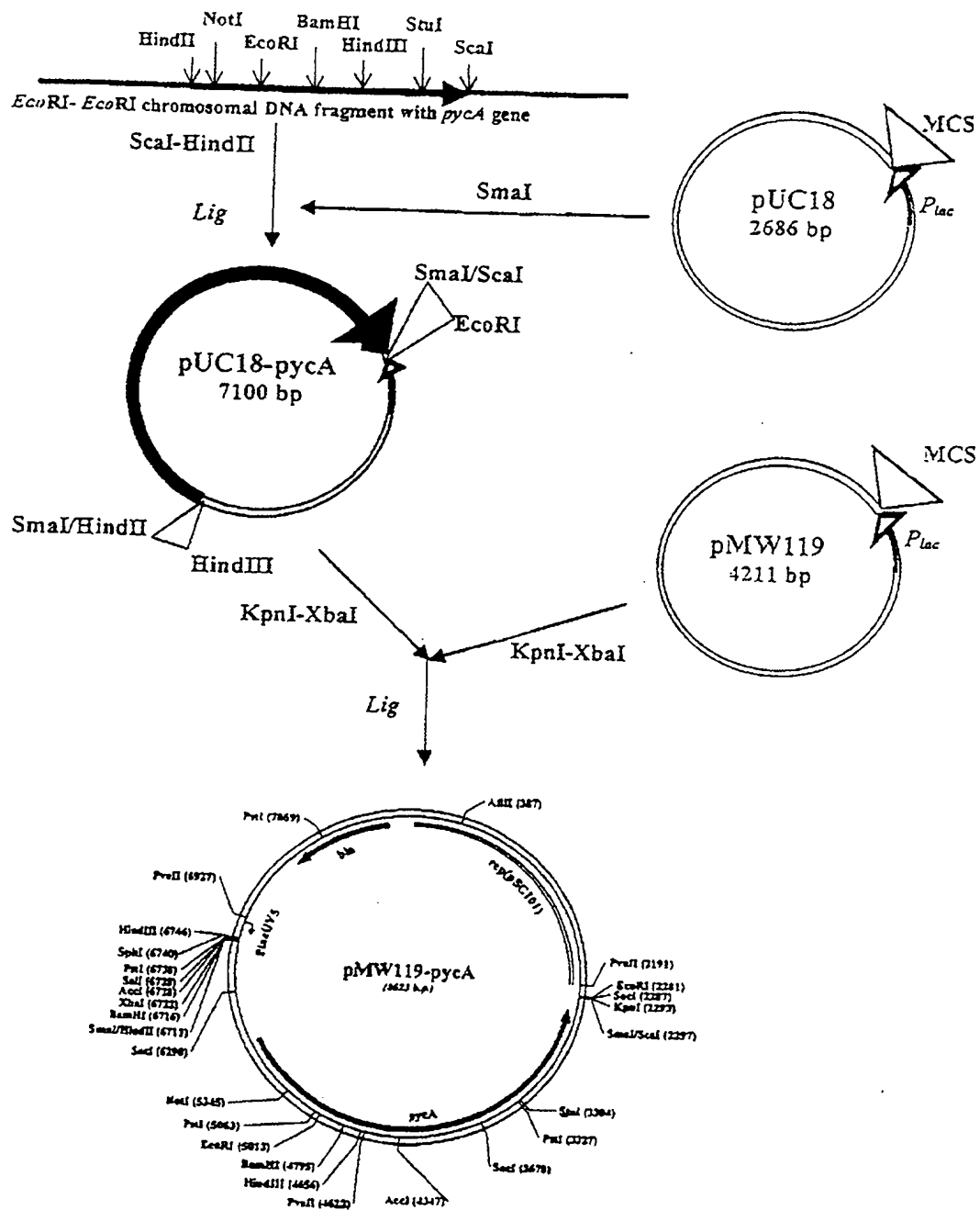


Fig. 3



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EUROPEAN SEARCH REPORT

Application Number
EP 00 12 1763

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Place of search THE HAGUE		Date of completion of the search 20 February 2001	Examiner van de Kamp, M
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons Δ : member of the same patent family, corresponding document	

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Place of search THE HAGUE		Date of completion of the search 20 February 2001	Examiner van de Kamp, M
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